Pest Manag Sci **57**:695–706 (2001)
DOI: 10.1002/ps.342

Viability and stability of biological control agents on cotton and snap bean seeds

Monica L Elliott,[1]* Elizabeth A Des Jardin,[1] William E Batson Jr,[2] Jacobo Caceres,[2] Philip M Brannen,[3] Charles R Howell,[4] D Michael Benson,[5] Kenneth E Conway,[6] Craig S Rothrock,[7] Raymond W Schneider,[8] Bonnie H Ownley,[9] Craig H Canaday,[10] Anthony P Keinath,[11] Donald M Huber,[12] Donald R Sumner,[13] Carl E Motsenbocker,[8] Peggy M Thaxton,[14] Marc A Cubeta,[15] Pamela D Adams,[16] Paul A Backman,[16] Julius Fajardo,[16] Melvin A Newman[10] and Roberto M Pereira[9]

[1]*University of Florida, Fort Lauderdale, FL 33314, USA*
[2]*Mississippi State University, Mississippi State, MS 39762, USA*
[3]*formerly Gustafson, Inc, McKinney, TX 77070, USA*
[4]*USDA-ARS, College Station, TX 77845, USA*
[5]*North Carolina State University, Raleigh, NC 27695, USA*
[6]*Oklahoma State University, Stillwater, OK 74078, USA*
[7]*University of Arkansas, Fayetteville, AR 72701, USA*
[8]*Louisiana State University, Baton Rouge, LA 70803, USA*
[9]*University of Tennessee, Knoxville, TN 37996, USA*
[10]*University of Tennessee, Jackson, TN 38301, USA*
[11]*Clemson University, Charleston, SC 29414, USA*
[12]*Purdue University, West Lafayette, IN 49707, USA*
[13]*University of Georgia, Tifton, GA 31793, USA*
[14]*Texas A&M University, College Station, TX 77843, USA*
[15]*North Carolina State University, Plymouth, NC 27962, USA*
[16]*Auburn University, Auburn, AL 36849, USA*

Abstract: Cotton and snap bean were selected for a multi-year, multi-state regional (south-eastern USA) research project to evaluate the efficacy of both commercial and experimental bacterial and fungal biological control agents for the management of damping-off diseases. The goal for this portion of the project was to determine the viability and stability of biological agents after application to seed. The biological seed treatments used included: (1) *Bacillaceae* bacteria, (2) non-*Bacillaceae* bacteria, (3) the fungus *Trichoderma* and (4) the fungus *Beauveria bassiana*. Seed assays were conducted to evaluate the following application factors: short-term (≤3 months) stability after seed treatment; quality (ie isolate purity); compatibility with chemical pesticides and other biocontrol agents; application uniformity between years and plant species. For the bacterial treatments, the *Bacillaceae* genera (*Bacillus* and *Paenibacillus*) maintained the greatest population of bacteria per seed, the best viability over time and the best application uniformity across years and seed type. The non-*Bacillaceae* genera *Burkholderia* and *Pseudomonas* had the least viability and uniformity. Although *Beauveria bassiana* was only evaluated one year, the seed fungal populations were high and uniform. The seed fungal populations and uniformity for the *Trichoderma* isolates were more variable, except for the commercial product T-22. However, this product was contaminated with a *Streptomyces* isolate in both the years that it was evaluated. The study demonstrated that *Bacillaceae* can be mixed with *Trichoderma* isolates or with numerous pesticides to provide an integrated pest control/growth enhancement package.

Keywords: *Arthrobacter*; *Bacillus*; *Beauveria bassiana*; *Burkholderia*; *Gossypium hirsutum*; *Paenibacillus*; *Phaseolus vulgaris*; *Pseudomonas*; seed treatments; *Trichoderma*

1 INTRODUCTION

Rhizoctonia solani Kühn and various *Pythium* species are important soil-borne pathogens that cause damping-off of seedlings, either before or after seedlings emerge from the soil, resulting in reduced plant stands and delayed emergence. Since these pathogens infect

* Correspondence to: Monica L Elliott, University of Florida, Fort Lauderdale Research and Education Center, 3205 College Avenue, Fort Lauderdale, FL 33314-7799, USA
E-mail: melliott@ufl.edu
Contract/grant sponsor: USDA, CSREES, Southern Region; contract/grant number: IPM 97-34103-5036
(*Received 26 October 2000; accepted 26 March 2001*)

in the earliest stages of plant growth, protection must be initiated when the seed is planted. Chemical seed treatments are currently the primary method used to manage damping-off. However, this is an ideal pathosystem in which to implement biological control.[1–3] The biological treatment does not need to survive and thrive for the entire growing season; it need only be effective during stand establishment. Since the biological treatment may be applied directly to seed, the organism is immediately available in the infection court for seedling protection. Therefore, a biological seed treatment for damping-off disease must be present and viable when the seed is planted. To be successful, a commercial product based on a biological agent must be consistently viable from year to year at each planting location where it is used.

Cotton and snap bean, representing a field and vegetable crop grown across the southern region of the USA, were selected for a multi-year, multi-state regional research project to evaluate the efficacy of both commercial and experimental bacterial and fungal biological control agents for the management of damping-off diseases. Both crops are susceptible to these diseases.[4–6] As part of this project, seed assays were conducted to determine viability, purity and concentration of each biological control treatment. Seed assays were conducted at the time the treated seed was delivered to cooperators and/or when the seed was planted. The data presented herein is a summary of the seed assay data obtained during a 5-year period. Preliminary data has been published for some of the results presented.[7–10]

2 EXPERIMENTAL METHODS

2.1 Seed treatments

Cotton (*Gossypium hirsutum* L cv 'Deltapine 50') and snap bean (*Phaseolus vulgaris* L var *vulgaris* cv 'Strike') seeds were treated either in bulk or on-site. Cotton seed was acid delinted and neutralized prior to treatment. Individual cooperators did bulk treatment of seed with their putative bacterial or fungal biocontrol agents. Bulk treated seed was then shipped to the project coordinator (W Batson) in Mississippi who packaged each treatment into 100-seed lots for delivery to planting site cooperators in each state. On-site treatment of seed was done by each planting site cooperator just prior to planting, using formulations of the bacterial or fungal isolates supplied by the company or cooperator who had formulated the biocontrol agent.

The seed treatments used can be divided into four main groups: (1) *Bacillaceae* bacteria, *Bacillus* and *Paenibacillus*, which are Gram-positive endospore-forming bacteria; (2) non-*Bacillaceae* bacteria, which include non-spore-forming Gram-positive and Gram-negative bacteria; (3) the fungus *Trichoderma*; (4) the fungus *Beauveria bassiana* (Balsamo) Vuill. Eight bacterial and eight fungal agents were evaluated at least twice during the 5-year study. All other biological agents were evaluated once.

2.2 Commercially-formulated bacterial and fungal biocontrol agents

The following bacterial isolates were supplied by Gustafson, Inc, Plano, TX, USA: *Bacillus subtilis* GB03 (Kodiak®), *Paenibacillus pabuli* GB51, *Bacillus cereus* GB37 (UW85),[11] *B macerans* GB32, *B subtilis* GB29 (ASC66570), *B pumilus* GB34, *Bacillus* sp GB35, and *Paenibacillus macerans* GB49. Rates for cotton seed are listed in Table 1 and those for snap bean seed in Table 2. The carrier used to formulate the *Bacillaceae* products was applied at $0.15\,g\,kg^{-1}$ seed. Gustafson also supplied an isolate of *Trichoderma harzianum* Rifai that was applied at $\log_{10}$ CFU per g of seed of 4.3 in 1996 and 4.6 in 1997. The rates were the same for both cotton and snap bean seed.

Trichoderma harzianum Rifai strain KRL-AG2 (T-22 Planter Box, BioWorks, Inc, Geneva, NY) was applied as a dry powder ($1.0 \times 10^7\,CFU\,g^{-1}$ dry weight) by each planting site cooperator immediately before planting.[12] The application rate was $\log_{10}$ $CFU\,g^{-1}$ seed of 4.6 in 1997 and 4.7 in 1998 for cotton seed, and $\log_{10}$ $CFU\,g^{-1}$ seed of 4.5 in 1997 and 4.6 in 1998 for snap bean.

Burkholderia cepacia type Wisconsin (Deny®, CCT Corp, Shawnee, KS) was applied by each planting site cooperator immediately before planting. The cotton or snap bean seed was moistened by misting and then mixed with Deny ($10^5\,CFU\,g^{-1}$ dry weight formulation). Rates are listed in Tables 3 and 4.

Vitazyme (Vital Earth Resources, Gladewater, TX) is a mixture of 15 bacteria, including five Gram-positive cocci, seven Gram-positive rods, and three Gram-negative rods. One of the Gram-positive rods is in the family *Bacillaceae* (*P macerans*). Vitazyme contained $2.0–7.0 \times 10^6\,CFU\,ml^{-1}$. Seed were soaked for 5 min in a 10% Vitazyme solution and then air dried.

2.3 Non-commercial bacterial biocontrol agents

The non-commercial bacterial isolates were supplied by various individuals, as described below. Prepared media or media components were obtained from Difco Laboratories (Detroit, MI), Fisher Scientific (Pittsburgh, PA) or Sigma (St Louis, MO).

Arthrobacter globiformis A180R and *Pseudomonas fluorescens* BD4-13 (supplied by C Rothrock, University of Arkansas)[13] were grown on 1/3-strength tryptic soy agar for 1–2 days, then used to start liquid cultures in 200 ml of Luria-Bertania broth [tryptone ($10\,g\,liter^{-1}$), yeast extract ($5\,g\,liter^{-1}$) and sodium chloride ($10\,g\,liter^{-1}$)] for *Pseudomonas* and 1/3-strength tryptic soy broth for *Arthrobacter*. Flasks were incubated on a shaker at $120\,rev\,min^{-1}$ for 48 h at room temperature (20–22 °C). Liquid cultures were centrifuged at 5016 *g* in 1995 and 1996 and 3560 *g* in 1997–99 for 20 min; broth was decanted. The pelleted bacterial cells were resuspended in phosphate buffer

(0.02 M; pH 7.0), centrifuged, and resuspended in buffer. In 1995 and 1996, concentrated bacterial suspensions were shipped to each cooperator, who treated seed prior to planting. In 1997–1999, seed were bulk treated in Arkansas by mixing a bacterial suspension with the seed, and then air dried. Rates are listed in Tables 3 and 4.

Burkholderia cepacia 5.5B (supplied by M Benson, North Carolina State University)[14] was grown on potato dextrose agar for 48 h. A portion of a colony was transferred to a 500-ml flask containing nutrient broth adjusted to pH 5.8 with hydrochloric acid (0.1 M) and incubated with shaking at 150 rev min^{-1} at room temperature. After 4 days, cultures had developed a purple color, indicating the production of phenazine.[15] Cultures were centrifuged at 3250 *g* for 30 min, and bacterial cells were resuspended in methyl cellulose (20 g $liter^{-1}$; 50 ml; Sigma, M-6385, 25 centipoises). The bacterial preparation was applied to seed immediately after resuspension. Multiple batches of 460 g of seed were treated by dripping five 10-ml amounts of the 1×10^{11} CFU ml^{-1} bacterial suspension over the seed and stirring until all seeds were moist. Seeds were placed on a metal sheet and dried for 4 h in a laminar flow hood. Treated seeds were stored at 4 °C until shipment by overnight delivery to the cooperator in Mississippi.

A seed treatment consisting of a mixture of three *Bacillus megaterium* strains (91-2, 91-5, 91–114) was supplied by R Schneider, Louisiana State University. Each strain was grown individually on nutrient agar for 48 h at room temperature. For each isolate, a suspension was made by washing cultures from 65 plates with phosphate buffer (25 mM; pH 6.8) and adjusting to an absorbance of 2.0 at a wavelength of 600 nm. The suspension was mixed with sterilized talc at a ratio of 1500 ml suspension to 3000 cm^3 talc and allowed to dry for 2 days with occasional stirring. Dried talc formulations of each of three isolates of *B megaterium* were prepared separately and then combined in a 1:1:1 ratio. Approximately 1 kg of seeds were moistened with 83 ml of a 1.1% solution of guar gum prepared with 10% L-arabinose, then 600 cm^3 of the dried talc inoculum was added to the moistened seed. The entire mixture was shaken in a plastic box for 1 min to coat the seeds, which were then dried under moving air for at least 24 h. Excess talc inoculum was removed by shaking the seeds over a 1-mm sieve.

Bacillus subtilis strains BA101 and E69 (supplied by B Ownley, University of Tennessee) were grown in Minimal 3-C broth (1 liter) in a 2-liter culture flask on a rotary shaker (100 rev min^{-1}) at 25–30 °C for 60–72 h.[16] Broth cultures were centrifuged at 10 000 rev min^{-1} for 10 min (Sorvall RC-5B centrifuge, GSA rotor). The supernatant was discarded and the bacterial pellets were resuspended in methyl cellulose solution (20 g $liter^{-1}$). The bacterial-methyl cellulose suspension was mixed with snap bean or cotton seed at 500 ml kg^{-1} seed. The treated seeds were placed under a laminar flow hood and air-dried. Treated seeds were stored at 4 °C until shipment by overnight delivery to the cooperator in Mississippi.

2.4 Non-commercial fungal biocontrol agents

Trichoderma harzianum OK-110 (supplied by K Conway, Oklahoma State University) was grown in molasses-bran medium in a Hi-Density Lab-Line Fermentor System (Melrose Park, IL).[17,18] After 1 week, mycelial fragments and conidia were collected on a 500-μm sieve, spread on waxed paper, dried and ground (Glen Mills Co, Maywood, NJ). This biomass (0.2 g) was added to carboxymethyl cellulose (20 g $liter^{-1}$) and then applied to 1 kg of seed.

Beauveria bassiana isolate 11.98 (supplied by R Pereira, University of Tennessee) was grown on Sabouraud dextrose agar plus yeast extract (5 g $liter^{-1}$) at 23 °C for a minimum of 15 days. Conidia were scraped from the surface of the agar plates with a rubber spatula and passed through a 100-μm sieve to eliminate mycelial and agar fragments. Conidia were then suspended in methyl cellulose solution (20 g $liter^{-1}$) at 20 mg conidia ml^{-1} of suspension. The conidial suspension was mixed with cotton or snap bean seed at 500 ml kg^{-1} seed. Treated seeds were air-dried under a laminar flow hood.

2.5 Biological and chemical seed treatment mixtures

Eight isolates of *Trichoderma virens* (Miller, Giddens & Foster) were supplied by C Howell, USDA-ARS, College Station, Texas. Isolates are listed in Table 5. Inoculum for each isolate was produced in a medium of 5% ground millet (1995–96) or wheat bran (1997–99) and 1% ground peat moss in deionized water. Since these isolates were known not to be effective against *Pythium* spp, the following treatment procedure was followed.[19] Seed was first treated with metalaxyl (0.5 ml Apron FL kg^{-1} seed), and then coated with a latex sticker before applying the air-dried, ground fungal treatment (7% w/w, ~55 g kg^{-1} seed). A previous study had demonstrated that there was no negative effect of the fungicide on the *Trichoderma* strains.[19] Some treatments were a mixture of one of these *Trichoderma* isolates with *Paenibacillus* GB49. The bacterial isolate was applied in an aqueous slurry with the metalaxyl, prior to application of the fungal isolate.

In 1998, two snap bean treatments, provided by C Canaday, University of Tennessee, consisted of *Bacillus* GB03, at two different rates, mixed with captan (1.25 g Captan 400-D kg^{-1} seed). In 1999, additional snapbean treatments provided by Gustafson, Inc consisted of *Bacillus* GB03 or *Paenibacillus* GB49 mixed with the following chemicals (rate per kg of seed): thiram (1.25 g GUS-42S), quintozene (1.3 ml RTU-PCNB), metalaxyl (0.5 ml Allegiance), streptomycin (0.55 g AgStrep-50) and chlorpyrifos (1.25 g Lorsban 50).

Also in 1999, the three Gustafson bacterial treatments applied on cotton seed were used only as

mixtures with the chemical seed treatments carboxin–quintozene (3.9 ml Vitavax-PCNB kg^{-1} seed), and metalaxyl (0.5 ml Allegiance kg^{-1} seed).

2.6 Seed inoculum density assessment

When bulk-treated seeds were shipped by overnight delivery to cooperators, treated seeds were also shipped to M Elliott, University of Florida, for quantifying bacteria and fungi on the seed. Four replicates of each biological seed treatment and the non-treated control were assayed on 13 April 1995, 3 April 1996, 4 April 1997, 8 April 1998 and 25 March 1999. For each replicate, five seeds were placed in sterile saline phosphate buffer [NaCl (8.5), $K_2PO_4 \cdot 3H_2O$ (11.4) and KH_2PO_4 (6.8 g $liter^{-1}$); pH 6.8; 5 ml], sonicated for 30 s in an ultrasonic bath and soaked for 30 min. Suspensions from seeds treated with fungal biocontrol agents, with or without chemical pesticides, were surface plated (0.1 ml) by serial dilution on 1/5-strength potato dextrose agar (1/5 PDA). In 1995 and 1996 only, the medium was amended with streptomycin sulfate (100 μg ml^{-1}). Suspensions from seeds treated with bacterial agents, with or without chemical pesticides, were surface plated (0.1 ml) by serial dilution on unamended, solidified 1/3-strength tryptic soy broth (1/3 TSBA). Suspensions from untreated control seeds were duplicate plated on unamended 1/5 PDA and 1/3 TSBA. Suspensions from seeds treated with a mixture of fungal and bacterial isolates were duplicate plated on 1/5 PDA amended with streptomycin sulfate (100 μg ml^{-1}) and rifampicin (50 μg ml^{-1}), and 1/3 TSBA amended with cycloheximide (100 μg ml^{-1}). In 1998, suspensions from the *Trichoderma* T22 treatment were duplicate plated on unamended 1/5 PDA and 1/3 TSBA amended with cycloheximide (100 μg ml^{-1}). Cultures were incubated at 28 °C, and counts were made as appropriate for each biological treatment.

At the time that each cooperator planted seeds, an extra packet of seed from each biological treatment was also shipped (overnight delivery) to the University of Florida to determine the populations of bacteria or fungi remaining on the seed, using the method described previously. For treatments applied to bulk seed, two (1995 and 1996) or three (1997–99) replicates of each treatment were assayed. For treatments applied to seeds at planting by the cooperator (eg *T harzianum* T-22, *B cepacia* Deny), four replicates (1996) or three replicates (1997–99) were assayed. The last dates for seed assays were 18 May 1995, 13 June 1996, 16 May 1997, 22 May 1998 and 18 May 1999. This represents an average of 49 days after the first assay at delivery, with a range of 35–71 days.

2.7 Statistical analysis

Analysis of variance was performed using SAS procedure GLM (SAS version 6.12, SAS Institute, Inc, Cary, NC) for each individual seed treatment in each year, across all planting locations. The coefficient of variation was determined to compare variation of planting population means between years for individual isolates or between isolates. When appropriate, means for seed populations values at delivery or at planting were compared across years for individual seed treatments using the LSD *t*-test. Using the Dunnett's *t*-test, means for seed population values at planting for each planting location were compared against mean seed population values at delivery for each individual seed treatment in each year. From these results, the percentage of planting locations that had planting mean values significantly different ($P = 0.05$) from the delivery seed value could be calculated.

3 RESULTS

3.1 Non-treated seed

Fungi and bacteria naturally associated with cotton and snap bean seed were determined in all years. Only in 1995 were any fungi detected on cotton seed, at 0.7 $\log_{10}$ CFU per seed. Fungi were detected on snap bean seed only in 1999, at 0.3 $\log_{10}$ CFU per seed. In all 5 years, bacteria were detected from both seed types. In 1995 on cotton seed, the population was 1.8 $\log_{10}$ CFU per seed. For the other 4 years on cotton seed, the population was less than 1.0 $\log_{10}$ CFU per seed. Bacteria were detected in all years on snap bean seed at an average of 1.4 $\log_{10}$ CFU per seed.

3.2 *Bacillaceae* bacterial treatments

For the *Bacillus* and *Paenibacillus* treatments applied to cotton seed in bulk prior to delivery to cooperators, populations at delivery in all years ranged from 3.5 (GB35 in 1999) to 5.8 (E69 in 1999) $\log_{10}$ CFU per seed, with an overall average of 4.8 (Table 1). The average bacterial population per seed at planting, across all sites in all years, decreased only 0.2 $\log_{10}$ CFU per seed to 4.6. The percentage of sites that had bacterial populations on the seed at planting that were significantly different from the populations on the seed at delivery averaged only 12.8%, with six isolates having no differences between delivery and planting seed populations in at least one treatment year. For *Paenibacillus* strain GB49, there were no significant differences among mean bacterial population per seed across years, even though the 1999 cotton treatment was mixed with chemical fungicide seed treatments. One *Bacillus* treatment (GB03) was used also as a treatment applied by individual cooperators prior to planting in 1995 for cotton. Even this treatment had a mean bacterial population per seed, across all sites, of 4.7 $\log_{10}$ CFU per seed (Table 1).

All snap bean *Bacillaceae* seed treatments were applied in bulk prior to delivery to cooperators. Populations at delivery across all years ranged from 3.3 (*Bacillus* GB35 in 1998) to 6.2 (*Bacillus* BA101 in 1999) $\log_{10}$ CFU per seed, with an overall average of 4.7 (Table 2). The average bacterial population per seed at planting decreased only 0.2 $\log_{10}$ CFU per seed to 4.5. The percentage of sites that had seed bacterial

populations at planting that were significantly different from the populations on the seed at delivery averaged only 17.9%, with four isolates having no differences between delivery and planting populations in at least one treatment year.

The coefficient of variation, for seed populations at planting, for 17 of 21 cotton treatments and 17 of 19 snap bean treatments was less than 5%, and was less than 10% for the remaining treatments (Tables 1 and 2). This was true for the treatments provided by a commercial laboratory (Gustafson) and those provided by individual cooperators (Louisiana *Bacillus* mix and Tennessee *Bacillus* strains BA101 and E69). Isolates that were consistent among years (ie no significant differences at delivery) were GB03 and GB49 on cotton seed in 1997–1999, GB37 and GB49 on snap bean seed for all years evaluated, and GB29 on snap bean seed in 1997–1998. When means for bacterial seed populations at delivery were compared for each isolate in each year between seed types, there was a difference between seed types for only seven of the 21 comparisons (LSD *t*-test at $P = 0.05$). They were GB37 in 1996, GB37 and *B megaterium* Louisiana mix in 1997, GB29 in 1998, and GB03, GB49 and BA101 in 1999.

3.3 Non-*Bacillaceae* bacterial treatments

For those four non-*Bacillaceae* bacterial treatments

Year	Application rate (log_{10} CFU g^{-1} seed)	log_{10} CFU per seed			Site differences[c] (%)	CV[d] (%)
		Delivery[a]	Planting[b]	Range[b]		
Bacillus cereus GB37						
1996	7.2	4.0 h	3.4 h	2.8–3.8	22	8.5
1997	7.2	4.9 g	4.6 g	4.5–4.9	9	3.5
Bacillus megaterium Louisiana mix						
1997	ND[e]	5.3 g	4.7 h	4.1–5.0	73	3.3
1998	ND	5.0 h	4.9 g	4.7–5.0	8	1.6
Bacillus subtilis GB03 (Kodiak®)						
1995	6.3	NA[f]	4.7 g	2.0–5.5	NA	1.9
1996	6.3	4.3 h	4.0 h	3.7–4.4	22	4.3
1997	6.3	4.9 g	4.7 g	4.5–4.9	0	3.1
1998	6.3	4.8 g	4.8 g	4.7–4.9	0	2.2
1999	6.3	5.0 g	4.8 g	4.6–5.0	20	3.4
Bacillus subtilis GB29 (ASC66570)						
1996	7.2	4.3 j	4.1 j	3.5–4.5	0	7.4
1997	7.2	4.7 h	4.7 h	4.4–4.9	0	4.0
1998	7.2	5.2 g	4.9 g	4.8–5.2	25	2.5
Paenibacillus macerans GB49						
1997	7.2	4.9	4.7	4.6–4.9	18	3.0
1998	7.2	4.8	4.8	4.6–4.9	0	2.7
1999	7.2	4.9	4.8	4.5–5.1	10	3.6
Bacillus sp GB35						
1998	5.8	3.5	3.2	3.0–3.5	8	7.1
Bacillus pumilus GB34						
1999	7.2	4.1	4.1	3.9–4.1	0	3.7
Bacillus subtilis BA101						
1999	ND	5.6	5.5	5.4–5.7	0	2.5
Bacillus subtilis E69						
1999	ND	5.8	5.6	5.3–5.7	30	2.3
Paenibacillus macerans GB32						
1996	7.2	4.4	4.1	3.6–4.6	11	6.8
Paenibacillus pabuli GB51						
1998	7.2	5.0	4.8	4.7–5.0	0	2.9

Table 1. *Bacillaceae* bacterial treatments applied to cotton seed, either in bulk before shipping to planting locations or just prior to planting

[a] Mean value (four replicates) at delivery of bulk treated seed to cooperators. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[b] Mean value and range at planting across all locations in each year; 1995 = five locations; 1996 = nine locations, 1997 = 11 locations; 1998 = 12 locations; 1999 = 10 locations. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[c] Percentage of locations that had seed mean values at planting that were significantly different ($P = 0.05$) from those at delivery of the bulk treated seed, according to Dunnett's *t*-test.

[d] Coefficient of variation for seed population values at planting.

[e] ND, not determined. See text for application details.

[f] NA, not applicable. Seed treated by individual cooperators just prior to planting.

applied to cotton seed in bulk prior to delivery to cooperators (*Pseudomonas, Burkholderia cepacia* 5.5b and OK1, Vitazyme), populations at delivery across all years, ranged from 0 to 4.1 $\log_{10}$ CFU per seed, with an overall average of 0.8 (Table 3). The percentage of sites that had seed bacterial populations at planting that were significantly different from the populations on the seed at delivery averaged 19.5%. However, this number should be interpreted carefully, since the Vitazyme and Gram-negative bacterial treatments were either not detected at delivery or at planting for a majority of sites. Across all non-*Bacillaceae* treatments, whether bulk treated or treated on site, the average bacterial population at planting was 1.1 $\log_{10}$ CFU per seed.

For the three non-*Bacillaceae* bacterial treatments applied to snap bean seed in bulk prior to delivery to cooperators (*Pseudomonas, Burkholderia cepacia* 5.5b, Vitazyme), populations at delivery across all years ranged from 0 to 4.9 $\log_{10}$ CFU per seed, with an overall average of 1.9 (Table 4). The percentage of sites that had seed bacterial populations at planting that were significantly different from the populations on the seed at delivery averaged 51.2%. Across all treatments, whether bulk treated or treated on site, the average bacterial population at planting was 1.5 $\log_{10}$ CFU per seed. As with the cotton seed treatments, applying bacterial treatments to the seed just prior to planting did not necessarily increase the bacterial seed populations at planting.

The non-*Bacillaceae* bacterial treatments for both seed types also had very large coefficients of variation for seed populations at planting, often greater than 100%, indicating extreme variability in bacterial populations among replications of each treatment at each site. The average coefficient of variation for all non-*Bacillaceae* bacterial treatments was 120% for cotton and 95% for snap bean (Tables 3 and 4).

Year	Application rate ($\log_{10}$ CFU g^{-1} seed)	$\log_{10}$ CFU per seed			Site differences[c] (%)	CV[d] (%)
		Delivery[a]	Planting[b]	Range[b]		
Bacillus cereus GB37						
1996	7.2	4.4	3.8 h	3.3–4.2	22	8.6
1997	7.2	4.7	4.3 g	3.8–4.9	10	5.0
Bacillus megaterium Louisiana mix						
1997	ND[e]	4.1 h	3.8 h	3.2–4.1	40	3.7
1998	ND	5.0 g	4.9 g	4.7–5.0	0	2.3
Bacillus subtilis GB03 (Kodiak®)						
1996	6.3	4.2 j	4.0 j	3.6–4.4	25	3.7
1997	6.3	5.0 g	4.8 g	4.6–5.1	0	4.0
1998	6.3	4.5 h	4.5 h	4.3–4.9	10	3.4
1999	6.3	4.3 hj	4.1 j	3.8–4.5	11	5.2
Bacillus subtilis GB29 (ASC66570)						
1996	7.2	4.4 h	4.2 j	3.7–4.6	13	3.7
1997	7.2	5.0 g	4.6 h	4.0–4.9	67	2.6
1998	7.2	4.9 g	4.9 g	4.7–5.1	0	3.3
Paenibacillus macerans GB49						
1997	7.2	5.1	4.5 h	4.3–4.8	44	7.1
1998	7.2	4.9	4.8 g	4.4–5.0	9	3.1
1999	7.2	4.5	4.5 h	4.0–4.8	11	4.6
Bacillus sp GB35						
1998	5.8	3.3	3.2	2.9–3.4	9	4.0
Bacillus subtilis BA101						
1999	ND	6.2	6.1	5.9–6.2	11	1.8
Bacillus subtilis E69						
1999	ND	6.0	5.9	5.7–6.0	0	2.4
Paenibacillus macerans GB32						
1996	7.2	4.4	4.2	3.6–4.6	22	3.1
Paenibacillus pabuli GB51						
1998	7.2	5.1	4.9	4.7–5.1	36	3.0

Table 2. *Bacillaceae* bacterial treatments applied to snap bean seed in bulk before shipping to planting locations

[a] Mean value (four replicates) at delivery of bulk treated seed to cooperators. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[b] Mean value and range at planting across all locations in each year; 1996 = nine locations; 1997 = 10 locations; 1998 = 11 locations; 1999 = nine locations. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[c] Percentage of locations that had seed mean values at planting that were significantly different ($P = 0.05$) from those at delivery of the bulk treated seed, according to Dunnett's *t*-test.

[d] Coefficient of variation for seed population values at planting.

[e] ND, not determined. See text for application details.

Year	Application rate (log_{10} CFU g^{-1} seed)	log_{10} CFU per seed: Delivery[a]	Planting[b]	Range[b]	Site differences[c] (%)	CV[d] (%)
Arthrobacter globiformis A180R						
1995	ND[e]	NA[f]	3.9g	0–6.1	NA	14
1996	ND	NA	0.6h	0–2.2	NA	214
Pseudomonas fluorescens BD4-13						
1995	ND	NA	2.4g	0–3.7	NA	48
1997	9.7	4.1g	2.6g	0.1–4.2	60	11
1998	8.4	0h	0.1h	0–0.6	8	161
1999	8.9	0.8h	0.3h	0–0.9	0	200
Burkholderia cepacia Deny®						
1996	2.5	NA	0.2g	0–0.9	NA	123
1997	3.8	NA	0.5g	0–2.9	NA	40
Burkholderia cepacia 5.5b						
1997	10.0	0	0.1	0–0.5	9	278
Burkholderia cepacia OK1						
1998	ND	0	0.6	0–1.8	0	187
Vitazyme bacterial mix						
1999	2.5	0	1.2	0–4.0	40	45

[a] Mean value (four replicates) at delivery of bulk treated seed to cooperators. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[b] Mean value and range at planting across all locations in each year; 1995 = five locations; 1996 = nine locations, 1997 = 11 locations; 1998 = 12 locations; 1999 = 10 locations. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[c] Percentage of locations that had seed mean values at planting that were significantly different ($P = 0.05$) from those at delivery of the bulk treated seed, according to Dunnett's *t*-test.

[d] Coefficient of variation for seed population values at planting.

[e] ND, not determined. See text for application details.

[f] NA, not applicable. Seed treated by individual cooperators just prior to planting.

Table 3. Non-*Bacillaceae* bacterial treatments applied to cotton seed, either in bulk before shipping to planting locations or just prior to planting

Year	Application rate (log_{10} CFU g^{-1} seed)	log_{10} CFU per seed: Delivery[a]	Planting[b]	Range[b]	Site differences[c] (%)	CV[d] (%)
Pseudomonas fluorescens BD4-13						
1997	8.9	4.9g	3.3g	0.9–3.9	100	8
1998	8.0	0j	0.9h	0–2.4	45	43
1999	8.3	1.0h	0.1j	0–0.4	89	144
Burkholderia cepacia Deny®						
1996	2.5	NA[f]	0.1	0–0.5	NA	306
1997	3.8	NA	0.4	0–2.5	NA	21
Burkholderia cepacia 5.5b						
1997	10.0	1.8	0.9	0–2.3	0	131
Arthrobacter globiformis A180R						
1996	ND[e]	NA	6.0	3.6–7.1	NA	5
Vitazyme bacterial mix						
1999	2.3	1.8	0.5	0–1.3	22	101

[a] Mean value (four replicates) at delivery of bulk treated seed to cooperators. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[b] Mean value and range at planting across all locations in each year; 1996 = nine locations, 1997 = 10 locations; 1998 = 11 locations; 1999 = nine locations. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[c] Percentage of locations that had seed mean values at planting that were significantly different ($P = 0.05$) from those at delivery of the bulk treated seed, according to Dunnett's *t*-test.

[d] Coefficient of variation for seed population values at planting.

[e] ND, not determined. See text for application details.

[f] NA, not applicable. Seed treated by individual cooperators just prior to planting.

Table 4. Non-*Bacillaceae* bacterial treatments applied to snap been seed, either in bulk before shipping to planting locations or just prior to planting

3.4 *Beauveria* and *Trichoderma* fungal treatments

The *B bassiana* isolate was applied in bulk to both cotton and snap bean seed prior to delivery to cooperators for planting in 1999. There were 5.9 $\log_{10}$ CFU per seed at delivery for both cotton (Table 5) and snap bean (Table 6). Across all planting sites, there was an average 5.8 $\log_{10}$ CFU per seed for cotton and 6.0 $\log_{10}$ per seed for snap bean. No cotton site, but 33% of the snap bean sites, had fungal seed populations at planting that were significantly different from the seed population at delivery.

All *Trichoderma* fungal treatments except for one (T22 Planter Box) were applied in bulk to both cotton and snap bean seed prior to delivery to cooperators. *Trichoderma* T22 Planter Box was used as a seed treatment applied by individual cooperators just prior

Table 5. Fungal treatments applied to cotton seed, either in bulk before shipping to planting locations or just prior to planting

	$\log_{10}$ CFU per seed				
Year	*Delivery*[a]	*Planting*[b]	*Range*[c]	*Site differences*[d] *(%)*	*CV*[e] *(%)*
Beauveria bassiana					
1999	5.9	5.8	5.5–6.0	0	3.5
Trichoderma harzianum T-22 Planter Box					
1997	NA[f]	3.3	2.8–4.0	NA	4.2
1998	NA	3.5	2.9–4.1	NA	5.0
Trichoderma harzianum Gustafson					
1996	2.5 g	2.0 g	1.5–2.4	44	6.4
1997	0 h	0.2 h	0–1.0	36	71.4
Trichoderma harzianum OK110					
1995	0.9 h	0.6 h	0.2–1.0	20	39.8
1996	0 j	0 j	0	0	0
1999	4.4 g	4.3 g	4.1–4.5	0	5.1
Trichoderma virens G4					
1995	4.7 g	4.6 g	4.3–5.0	40	2.2
1996	2.9 j	2.3 j	2.0–2.8	56	6.2
1997	3.1 h	3.0 h	2.9–3.6	18	4.0
Trichoderma virens G6					
1995	4.1 g	3.8 g	3.6–4.2	40	0.9
1997	3.0 h	2.6 h	2.3–2.9	64	5.3
1998	2.8 h	2.6 h	2.3–2.9	8	6.8
1999	1.9 j	1.6 j	1.2–1.8	30	8.6
Trichoderma virens TV108					
1996	2.6	2.0 h	1.7–2.3	89	3.4
1997	2.6	2.3 g	2.0–2.7	36	6.9
Trichoderma virens TV109					
1996	2.9 g	2.3 g	2.0–3.0	78	4.4
1997	2.1 h	2.0 h	1.9–2.2	0	8.7
Trichoderma virens TV111					
1996	2.4 g	2.1 h	1.8–2.4	33	6.7
1997	3.1 g	2.8 g	2.6–3.1	27	4.8
1998	2.9 g	2.7 g	2.3–3.0	8	8.2
1999	1.4 h	1.4 j	0.9–1.8	0	34.9
Trichoderma virens and *Trichoderma koningii* fusant TV117					
1998	3.1 g	3.0	2.7–3.3	0	7.1
1999	3.0 h	2.9	2.6–3.0	0	6.7
Trichoderma virens TV110					
1996	3.0	2.3	1.9–2.8	89	4.0
Trichoderma virens TV116					
1998	2.8	2.6	2.2–2.8	33	5.0

[a] Mean value (four replicates) at delivery of bulk treated seed to cooperators. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[b] Mean value at planting across all locations in each year; 1995 = five locations; 1996 = nine locations, 1997 = 11 locations; 1998 = 12 locations; 1999 = 10 locations. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[c] Range of mean values for planting locations in each year.

[d] Percentage of locations that had seed mean values at planting that were significantly different ($P = 0.05$) from those at delivery of the bulk treated seed, according to Dunnett's *t*-test.

[e] Coefficient of variation for seed population values at planting.

[f] NA, not applicable. Seed treated by individual cooperators just prior to planting.

Table 6. Fungal treatments applied to snap bean seed, either in bulk before shipping to planting locations or just prior to planting

	log_{10} CFU per seed			*Site differences*[d]	*CV*[e]
Year	*Delivery*[a]	*Planting*[b]	*Range*[c]	*(%)*	*(%)*
Beauveria bassiana					
1999	5.9	6.0	5.1–6.2	33	1.9
Trichoderma harzianum T-22					
1997	NA[f]	2.8	2.1–3.8	NA	7.0
1998	NA	3.0	2.6–3.7	NA	4.0
Trichoderma harzianum OK110					
1996	1.4	1.9	1.7–2.4	11	12.8
Trichoderma harzianum Gustafson					
1996	2.1 g	2.1 g	2.0–2.4	0	11.9
1997	0.2 h	0.1 h	0–0.4	0	109.0

[a] Mean value (four replicates) at delivery of bulk treated seed to cooperators. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[b] Mean value at planting across all locations in each year; 1996 = nine locations, 1997 = 11 locations; 1998 = 12 locations; 1999 = 10 locations. For those treatments evaluated in multiple years, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[c] Range of mean values for planting locations in each year.

[d] Percentage of locations that had seed mean values at planting that were significantly different ($P = 0.05$) from those at delivery of the bulk treated seed, according to Dunnett's *t*-test.

[e] Coefficient of variation for seed population values at planting.

[f] NA, not applicable. Seed treated by individual cooperators just prior to planting.

to planting for both cotton and snap bean seed. For this treatment, the mean fungal population per seed, across all locations for each year, was not significantly different between years, with an average of 3.4 $\log_{10}$ CFU for cotton seed (Table 5) and 2.9 $\log_{10}$ CFU for snap bean seed (Table 6). Since we determined in 1997 that this commercial product was contaminated with a *Streptomyces* sp, assays were conducted for bacterial contamination in 1998. It was determined that the material present in the shipping container contained 8.5 $\log_{10}$ CFU of the fungus and 6.7 $\log_{10}$ CFU of the contaminating streptomycete per gram of material. The average seed populations for the streptomycete, across all treatment locations, was 2.0 $\log_{10}$ CFU per cotton seed and 1.8 $\log_{10}$ CFU per snap bean seed, a much higher ratio of streptomycete to *Trichoderma* than in the shipping container. The identity of the streptomycete was not determined.

For *Trichoderma* fungal treatments applied in bulk to cotton seed, populations at delivery across all years ranged from 0 (OK110 in 1996 and Gustafson in 1997) to 4.7 (G4 in 1995) $\log_{10}$ CFU per seed, with an overall average of 2.6 (Table 5). The average fungal population per seed at planting decreased only 0.3 $\log_{10}$ CFU for these treatments. The percentage of sites that had fungal seed populations at planting that were significantly different from populations on the seed at delivery averaged 31%.

Eight of the strains used to treat cotton were provided by one research group (USDA-ARS, Texas). For five of the six strains evaluated in multiple years (G4, G6, TV109, TV111, TV117), there were significant differences between years for fungal seed populations. In general, these populations decreased with succeeding years. Strain OK110, provided by the Oklahoma cooperator, had fungal seed populations that increased significantly in 1999 compared with previous testing years. In contrast, Gustafson's *Trichoderma* strain decreased significantly in fungal seed populations from 1996 to 1997.

Trichoderma fungal treatments applied in bulk to snap bean seed (OK110, Gustafson) had populations at delivery that ranged from 0.2 to 2.1 $\log_{10}$ CFU per seed, with an overall average of 1.2 (Table 6). The average fungal population per seed at planting was 1.4 $\log_{10}$ CFU. The percentage of sites that had fungal seed populations at planting that were significantly different from populations on the seed at delivery averaged 3.7%.

The coefficient of variation, for seed populations at planting, for 23 of 26 *Trichoderma* cotton seed treatments was <10% (Table 5), whereas for four of five *Trichoderma* snap bean seed treatments it was <13% (Table 6). The remaining treatments had extremely high coefficients of variation, 35% (TV111 on cotton seed in 1999), 40% (OK110 on cotton seed in 1995), 71% (Gustafson on cotton seed in 1997), and 109% (Gustafson on snap bean seed in 1997). Overall, the Texas strains were very stable within treatment years, but not between years.

3.5 Biological mixture treatments

Some cotton seed treatments were a mixture of a *Trichoderma* strain (TV111, TV116, TV117 or G6) with the *Paenibacillus* strain GB49 (data not shown). A mixture with *Trichoderma* strain G6 was evaluated three years and the other strains only once. For *Trichoderma* strain G6, the fungal population per seed was significantly increased ($P = 0.05$) by the addition of the bacterium GB49 in 1997 but not in 1998 and 1999. The seed population for G6 when mixed with GB49 was ($\log_{10}$ CFU per seed) 3.3, 2.7 and 1.9 in 1997, 1998 and 1999, respectively. No differences in seed populations were observed for the other *Trichoderma* strains when the *Paenibacillus* strain was added with them. Bacterial populations per seed were not affected by the addition of a fungal strain in any year.

3.6 Bacterial and chemical pesticide mixture treatments

Two *Bacillaceae* strains, *Bacillus* GB03 and *Paenibacillus* GB49, were mixed with chemical pesticides prior to their application to snap bean seed in addition to being evaluated alone. Two different application rates of the strains were used. For both strains, there was no negative effect from the addition of chemical pesticides (Table 7) and the seed populations at delivery and planting were increased significantly by the addition of chemicals in 1999. In addition, the eightfold increase in the normal GB03 and GB49 application rate

resulted in a similar increase on the seed, even with the addition of chemical pesticides.

4 DISCUSSION

Protecting seedlings from damping-off diseases is an ideal situation for biological control, since the biological agent needs to be effective only for a short time period. Furthermore, since the infection court for this disease is associated with the seed or newly emerged seedling, application of the biocontrol agent to the seed provides an efficient method for placing the biological agent in the appropriate location. In this study, the seed treatments evaluated included biological agents that were already commercially available or were being considered for commercial development. Application factors examined included short-term ($\leq$3 months) stability after seed treatment, quality (ie isolate purity), compatibility with chemical pesticides and with other biocontrol agents, and application uniformity between years and plant species.

The seed treatments used can be divided into four main groups: (1) *Bacillaceae* bacteria, *Bacillus* and *Paenibacillus*, which are Gram-positive endospore-forming bacteria; (2) non-*Bacillaceae* bacteria, which include non-spore-forming Gram-positive and Gram-negative bacteria; (3) the fungus *Trichoderma*; (4) the fungus *Beauveria bassiana*.

The greatest population of bacteria per seed and the best viability over time was associated with the *Bacillaceae* genera, both on cotton and snap bean seed. Even more important was the consistency between the bacterial seed populations obtained at delivery and those obtained when the seed was planted. Including both cotton and snap bean seed, for eight of the eleven *Bacillaceae* isolates no significant differences were found in bacterial seed populations between delivery and any planting location in one or more years for a total of 132 site years (number of sites per year multiplied by the number of years with no significant differences for each isolate). The other three *Bacillaceae* isolates (GB32, GB35 and GB37) had only one or two planting location seed populations that differed significantly from seed delivery populations. This consistency was achieved even though there were three different cooperators, two university and one commercial laboratory, providing these *Bacillaceae* isolates, each using a different carrier and application method for seed treatment.

Unfortunately, viability and consistency in application did not always result in disease control or increased plant stands.[7–9] For example: no biological seed treatment increased snap bean plant stand in 1996 at any location; only *P macerans* GB49 increased snap bean plant stand in 1997, and then only at two locations; only *P pabuli* GB51 increased snap bean plant stand at all locations in 1998. However, this could be due to the inability of the *Bacillaceae* seed treatments to control all the potential pathogens that may decrease plant stands. For example, the *Bacillus* strain GB03 will suppress *Rhizoctonia* and *Fusarium* spp but not necessarily *Pythium* spp.[20] Also, the plant growth-promoting activity of the biocontrol agents may not have been expressed until after our experiments were terminated.[20,21] Therefore, mixtures of biocontrol agents or mixtures of chemical pesticides and biocontrol agents may be required for complete plant protection or growth promotion.

Table 7. Snap bean seed treated with either a bacterial isolate alone, *Bacillus* GB03 or *Paenibacillus* GB49, or the bacterial isolate mixed with chemical pesticides

Isolate	Application rate (log_{10}CFU g^{-1} seed)	log_{10} CFU per seed				Site differences[e] (%)	CV[f] (%)
		Chemicals[a]	Delivery[b]	Planting[c]	Range[d]		
1998							
GB03	6.3	No	4.5 h	4.5 h	4.3–4.9	10	3.4
	6.3	Yes	4.6 h	4.6 h	4.4–4.7	0	2.2
	7.2	Yes	5.7 g	5.6 g	5.4–5.7	0	2.3
1999							
GB03	6.3	No	4.3 j	4.1 j	3.8–4.5	11	5.2
	6.3	Yes	4.7 h	4.8 h	4.4–5.0	11	2.7
	7.2	Yes	5.8 g	5.8 g	5.7–6.1	0	4.2
GB49	7.2	No	4.5 j	4.5 j	4.0–4.8	11	4.6
	7.2	Yes	4.8 h	4.8 h	4.4–4.9	11	2.3
	8.1	Yes	5.6 g	5.7 g	5.4–6.4	11	3.4

[a] Chemical pesticide used in 1998 was captan; chemical pesticides used in 1999 included thiram, quintozene, metalaxyl, streptomycin and chlorpyrifos.

[b] Mean value (four replicates) at delivery of bulk treated seed to cooperators. For each isolate in each year, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[c] Mean value at planting across all locations in each year; 1998 = 11 locations; 1999 = nine locations. For each isolate in each year, means followed by the same letter in the same column were not significantly different at $P = 0.05$, according to LSD *t*-test.

[d] Range of mean values for locations in each year.

[e] Percentage of locations that had seed mean values at planting that were significantly different ($P = 0.05$) from those at delivery of the bulk treated seed, according to Dunnett's *t*-test.

[f] Coefficient of variation for seed population values at planting.

This potential scenario was examined for two *Bacillaceae* isolates. The effects of the chemicals on the isolates were, if any, minimal. For example, in 1999 a chemical seed treatment that included one bactericide, three fungicides and one insecticide was added to both GB03 and GB49 without any deleterious effects. These observations are consistent with previous results.[22,23] Overall, these results demonstrate why, from a practical view point, *Bacillaceae* genera have been and continue to be a good choice for development as bacterial seed treatments.[3,20,24]

In contrast to the *Bacillaceae*, the lowest populations of bacteria and greatest amount of variability was associated with the non-*Bacillaceae* bacterial seed treatments, both on cotton and snap bean seed. This may be due to inappropriate formulations of the bacteria, since only Deny (*B cepacia*) and Vitazyme (bacterial mix) treatments were commercial products. However, the viability of these commercial materials was questionable. Deny was applied by cooperators just prior to planting, eliminating any decline in viability due to storage of treated seed. A packet of treated seed was shipped to the seed assay cooperator within 24h. In 1996 the seed assay cooperator determined the seed bacterial populations were less than 1.0 $\log_{10}$ CFU seed^{-1} and the planting cooperators were also asked to assay their containers of the Deny product for viability. The bacterium was found to be viable and the *B cepacia* population conformed to the label (data not shown). While the storage shelf-life of this product appeared to be adequate, its post-application shelf-life was minimal. This would be important information for the grower, as it is not uncommon for planting to be delayed (eg due to rainfall) after the seed and biological treatments are in the planter. A low post-application viability could also explain the disease control failure of this product in our study and others.[25]

Beauveria bassiana was only evaluated in one year, but the seed fungal values were high and uniform for both cotton and snap bean. While this fungus is normally associated with insect biocontrol, it has demonstrated antifungal activity. *B bassiana* has been shown to inhibit mycelial growth of several pathogens in vitro, including *Gaeumannomyces graminis* (Sacc) v Arx & J Olivier, *Fusarium oxysporum* Schlecht, *Armillaria mellea* (Vahl) Kummer, and *Rosellinia necatrix* Prill.[26,27] In greenhouse studies, *B bassiana* treatment reduced infection of onion bulbs by *F oxysporum* f sp *cepae*.[28]

The majority (eight of 13) of *Trichoderma* isolates were supplied by one cooperator (USDA-ARS, Texas). For these isolates, which were only evaluated on cotton, fungal seed populations at delivery were quite variable between years, despite the fact that the seed was formulated and applied similarly. Variability in seed populations at delivery and planting, within each year, for each isolate was initially high. For 1995 and 1996 data combined, the average percentage of planting sites that had fungal seed populations significantly different from those obtained at delivery was 60.7%. However, this was reduced to 18.7% for 1997 through 1999, with three of the isolates (TV109, TV111 and TV117) having treatment years when there was no significant difference between delivery seed populations and those at any planting location for a total of 43 site years. It is possible, but as yet unconfirmed, that the wheat bran used from 1997 to 1999 was a better substrate for these *Trichoderma* isolates than the millet seed used in 1995 and 1996. Four of these isolates were also mixed with *Paenibacillus* isolate GB49. The seed populations for these strains, fungal and bacterial, were not adversely affected.

The Gustafson and Oklahoma *Trichoderma* isolates had variable results between years. They were either present in consistently high seed populations, or detected at very low seed populations, if at all. *Trichoderma* OK110 was even variable between crops. It was consistently present on snap bean seeds in 1996, but not detected at all on cotton seeds in the same year. For all of these strains, these differences occurred despite using the same inoculum production method and the same level of inoculum.

In contrast, the only commercial formulation of *Trichoderma*, T-22 Planter Box, had consistent seed populations between years and across all planting locations for both cotton and snap bean seeds. This is significant since each cooperator applied this material just prior to planting. This is in sharp contrast to the commercial Gram-negative bacterial treatment (Deny) applied in a similar manner. Unfortunately, the T-22 Planter Box material had a quality control problem relative to purity. A streptomycete was detected in both years, even though different batches of product were used. This illustrates another problem that can be associated with scale-up of a biocontrol agent. The contaminant could be associated with the fungal isolate (ie impure isolate) or with the materials used to formulate the product. In either case, it demonstrates the necessity of a good quality control program through the entire production process.

Initial viability and stability of biocontrol agents are important first steps in assessing their use as seed treatments. This study illustrates achievements obtained and obstacles faced by researchers working with biological seed treatments. It is apparent that the endospore-forming *Bacillaceae* bacteria (eg *Bacillus* and *Paenibacillus* spp) will continue to have a clear advantage over Gram-negative bacteria, unless suitable formulation packages are developed for the latter group. A reliable formulation has been developed for *Trichoderma*, but purity issues still exist. The fact that these biocontrol agents can be mixed with each other and with numerous pesticides to provide a complete pest control/growth enhancement package is an excellent example of future integrated pest management systems. However, these systems must be both efficacious and economical for the producer.

ACKNOWLEDGEMENTS

This research was supported, in part, by USDA, CSREES, Southern Region IPM grant 97-34103-5036. Florida Agricultural Experiment Station Journal Series No R-08016.

REFERENCES

1 Paulitz T, Biological control of damping-off diseases with seed treatments, in *Biological Control of Plant Diseases: Progress and Challenges for the Future*, ed by Tjamos ES, Papavizas GC and Cook RJ, Plenum Press, New York, pp 145–156 (1992).

2 Taylor AG, Harman GE and Nielsen PA, Biological seed treatments using *Trichoderma harzianum* for horticultural crops. *HortTechnology* **4**:105–109 (1994).

3 Mathre DE, Cook RJ and Callan NW, From discovery to use: traversing the world of commercializing biocontrol agents for plant disease control. *Plant Dis* **83**:972–983 (1999).

4 Hendrix Jr FF and Campbell WA, Pythiums as plant pathogens. *Ann Rev Phytopath* **11**:77–98 (1973).

5 Watkins GM, *Compendium of Cotton Diseases*, American Phytopathological Society, St Paul, MN, USA (1981).

6 Sumner DR, Virulence of anastomosis groups of *Rhizoctonia solani* and *Rhizoctonia*-like fungi on selected germ plasm of snap bean, lima bean, and cowpea. *Plant Dis* **69**:25–27 (1985).

7 Batson WE Jr, Caceres J, Adams PD, Conway KE, Elliott ML, Huber DM, Keinath AP, Rothrock CS, Schneider RW, Molsenbocker CE and Sumner DR, Efficacy of biological seed treatments for control of the cotton seedling disease complex, 1996. *Biol Cult Tests* **12**:26–27 (1997).

8 Batson WE Jr, Caceres J, Backman P, Benson M, Brannen PM, Cubeta MA, Elliott ML, Huber DM, Keinath AP, Ownley B, Rothrock CS, Schneider RW, Molsenbocker CE, Sumner DR and Thaxton P, Efficacy of biological seed treatments for control of the cotton seedling disease complex, 1997. *Biol Cult Tests* **13**:21–22 (1998).

9 Batson WE Jr, Caceres J, Benson M, Brannen PM, Cubeta MA, Conway K, Elliott ML, Fajardo J, Huber DM, Keinath AP, Newman M, Ownley B, Rothrock CS, Schneider RW, Molsenbocker CE, Sumner DR and Thaxton P, Biological seed treatments for control of the seedling disease complex of cotton, 1998. *Biol Cult Tests* **14**:25–26 (1999).

10 Keinath A, Batson W Jr, Caceres J, Elliott M, Sumner D, Brannen P, Rothrock C, Huber D, Benson M, Conway K, Schneider R, Motsenbocker C, Cubeta M, Ownley B, Canaday C, Adams P, Backman P and Fajardo J, Evaluation of biological and chemical seed treatments to improve stand of snap bean across the southern United States. *Crop Prot* **19**:501–509 (2000).

11 Osburn RM, Milner JL, Oplinger ES, Smith RW and Handelsman J, Effect of *Bacillus cereus* UW85 on the yield of soybean at two field sites in Wisconsin. *Plant Dis* **79**:551–556 (1995).

12 Harman GE, Seed treatments for biological control of plant disease. *Crop Prot* **10**:166–171 (1991).

13 Hagedorn C, Gould WD and Bardinelli TR, Field evaluations of bacterial inoculants to control seedling disease pathogens on cotton. *Plant Dis* **77**:278–282 (1993).

14 Cartwright DK and Benson DM, Biological control of Rhizoctonia stem rot of poinsettia in polyfoam rooting cubes with *Pseudomonas cepacia* and *Paecilomyces lilacinus*. *Biol Control* **5**:237–244 (1995).

15 Cartwright DK, Chilton WS and Benson DM, Pyrrolnitrin and phenazine production by *Pseudomonas cepacia*, strain 5.5B, a biocontrol agent of *Rhizoctonia solani*. *Appl Microbiol Biotech* **43**:211–216 (1995).

16 Handelsman J, Rafefel S, Mester EH, Wunderlich L and Grau CR, Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. *Appl Environ Microbiol* **56**:713–718 (1990).

17 Papavizas GC, Dunn MT, Lewis JA and Beagle-Ristaino J, Liquid fermentation technology for experimental production of biocontrol fungi. *Phytopathology* **74**:1171–1175 (1984).

18 Conway KE, Maness NE and Motes JE, Integration of biological and chemical controls for Rhizoctonia aerial blight and root rot of rosemary. *Plant Dis* **81**:795–798 (1997).

19 Howell CR, DeVay JE, Garber RH and Batson WE, Field control of cotton seedling diseases with *Trichoderma virens* in combination with fungicide seed treatments. *J Cotton Sci* **1**:15–20 (1997).

20 Brannen PM and Kenney DS, Kodiak®—a successful biological-control product for suppression of soil-borne plant pathogens of cotton. *J Indust Microbiol Tech* **19**:169–171 (1997).

21 Raupach GS and Kloepper JW, Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* **88**:1158–1164 (1998).

22 Mahaffee WF and Backman PA, Effects of seed factors on spermosphere and rhizosphere colonization of cotton by *Bacillus subtilis* GB03. *Phytopathology* **83**:1120–1125 (1993).

23 Zhang J, Howell CR and Starr JL, Suppression of *Fusarium* colonization of cotton roots and Fusarium wilt by seed treatments with *Gliocladium virens* and *Bacillus subtilis*. *Biocontrol Sci Tech* **6**:175–187 (1996).

24 Emmert EAB and Handelsman J, Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiol Lett* **171**:1–9 (1999).

25 Zaki K, Misaghi IJ, Heydari A and Shatla MN, Control of cotton seedling damping-off in the field by *Burkholderia (Pseudomonas) cepacia*. *Plant Dis* **82**:291–293 (1998).

26 Renwick A, Campbell R and Coe S, Assessment of in vivo screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. *Plant Path* **40**:524–532 (1991).

27 Reisenzein H and Tiefenbrunner W, Growth inhibiting effect of different isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals) Vuill to plant parasitic fungi of the genera *Fusarium*, *Armillaria*, and *Rosellinia*. *Pflanzenschutzberichte* **57**:15–24 (1997).

28 Flori P and Roberti R, Treatment of onion bulbs with antagonistic fungi for the control of *Fusarium oxysporum* f sp *cepae*. *Difesa delle Piante* **16**:5–12 (1993).